42.11

Protection Against Borrelia burgdorferi Infection in Mice Immunized with Peptides from the Decorin-binding Adhesin Dbp

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Borrelia burgdorferi adhesion to host tissue is a key event in determining the fate of the initial bacterial challenge in the dermis and is a critical step in the progression of Lyme disease. Borrelia adherence to the proteoglycan decorin by two adhesins of the MSCRAMM family, decorin binding proteins A and B (DbpA and DbpB respectively) may be a critical step during the infection process. Vaccinations using DbpA have been successful in providing protection against infection with heterologous strains of B. burgdorferi. We previously demonstrated that DbpA contained 3 critical lysine residues that were critical for decorin binding. In this study, we demonstrated that DbpA-peptides containing these critical lysine residues interfered with decorin binding whereas peptides with chemically modified lysines or peptides spanning other regions of DbpA did not have affect decorin binding. Four different DbpA-peptides were tested for their ability to confer protection in a murine model of Lyme disease. These data suggest that vaccination using peptides incorporating critical binding domains of DbpA for decorin can confer a delayed-type hypersensitiviy response to DbpA and reduce the number of recoverable Borrelia from blood and joints of infected mice. Furthermore, arthritis incidence and the mean arthritis score was reduced in mice treated with DbpA-peptides.

42.12

Human dendritic cells discriminate between viable and killed *Toxoplasma gondii* tachyzoites: Implications in the generation of cell-mediated immunity against the parasite.

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We studied how the interaction between human dendritic cells (DC) and *Toxoplasma gondii* influences the generation of cell-mediated immunity against the parasite. We demonstrate that viable but not killed tachyzoites of *T. gondii* altered the phenotype of immature DC. DC infected with viable parasites upregulated expression of CD40, CD80, CD83, CD86 and HLA-DR and downregulated expression of CD115. These changes are indicative of DC maturation induced by *T. gondii*. DC maturation was not mediated by IL-1 β or TNF-α. Viable and killed tachyzoites had contrasting effects on cytokine production. DC infected with viable *T. gondii* rather than DC that phagocytosed killed parasites induced secretion of high amounts of IFN-γ by T cells from *T. gondii*-seronegative donors. This IFN-γ secretion was dependent in part on IL-12 production. IL-12-independent IFN-γ production was mediated by CD40-CD40 ligand and CD80/CD86-CD28 interactions. Taken together, *T. gondii*-induced DC maturation regulates T cell production of IFN-γ through release of bioactive IL-12 and through modulation of CD40 and CD80/CD86 signaling.

42.13

Effects of Mouse Malaria cDNA Library Transfection on the Antigen Presenting Cell Function

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Expression library immunization is one of the DNA vaccination methods and the library can [illegible] its DNA. We constructed cDNA library from erythrocytic stage of *Plasmodium berghei* and tested its efficacy for genetic immunization. Unexpectedly, the immunization of the library did not protect mice from lethal challenge of malaria parasites. There are two explanations for this result; partial expression of the library did not induce protective immunity and/or the expression of various parasite antigens, immunogenic, neutral, and immunosuppressive, might modify host antigen presenting cell function but competed with each other. If the latter is true, protective or suppressive plasmid(s) can be isolated by functional assay of antigen presenting cells. To test this idea, we divided the library into sublibraries and examined the antigen presenting ability of sublibrary-transfected splenic adherent cells. We found that some of the sublibrary-transfected cells showed higher antigen presenting ability and others showed lower ability than mock-transfected cells did. The results suggest that the transfection of the plasmids encoding malaria parasite antigens can modify host antigen presenting cell function.

42.14

ENHANCEMENT OF HEPATITIS C VIRUS CORE ANTIGEN-SEPCIFIC TPYE 1 T HELPER CELL RESPONSE BY RIBAVIRIN CORRELATES WITH THE INCREASED LEVEL OF IL-12.

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Combination IFN-α and ribavirin therapy of hepatitis C virus infected patients has been reported to improve the response rate to 50%. To further study the role [illegible] hepatitis C virus core protein with or without ribavirin. Forty C57BL/6 mice were immunized with core antigen once every two weeks and 0.5 mg ribavirin every day showed higher level of core-specific IgG2a as compared with those mice immunized with core antigen only. In addition, we found that *in vitro* recalled core antigen increased the levels of the T helper type 1 cytokines produced by spleen cells. In addition, the lipopolysaccharide (LPS)-stimulated peritoneal cells produced higher level of IL-12 in ribavirin-treated mice. The percentage of CD3$^+$ cells increased significantly both in spleen cells and peritoneal cells. Both the percentage and activity of natural killer cells were enhanced dramatically. The core-specific cytotoxic T cell activity also increased significantly. Thus, ribavirin may significantly promote the T helper type 1 immune response *in vivo*, furthermore, the effect of ribavirin on IL-12 level produced by LPS-stimulated peritoneal cells may contribute to the Th1 enhancing effect.

42.15

STRAIN-SPECIFIC DIFFERENCE IN PNEUMOCOCCAL POLYSACCHARIDE-SPECIFIC ANTIBODY-SECRETING CELL FREQUENCY CORRELATES WITH ANTBODY TITER.

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Bacterial polysaccharides (PS) typically behave as weak, T-independent immunogens in children and the elderly. While conjugation of PS to a carrier protein can enhance the PS-specific antibody (Ab) response, multivalent pneumococcal PS (PnPS) linked to identical carrier proteins (CRM_{197} : a non-toxic mutant of diphtheria toxin) exhibit significant serotype-specific variation in the PnPS-specific immunogenicity in humans. We have reported that different PnPS conjugated to CRM_{197} also vary in PS-specific immunogenicity in mice. Serotype 6B and 19F PS conjugated to CRM_{197} were capable of eliciting strong PnPS-and CRM_{197} -specific Ab responses. In contrast, immunization of CBA/J mice with PnPS, 23F, conjugated to CRM_{197} (23F- CRM_{197}) resulted in low to undetectable levels of PnPS-specific Ab despite levels of CRM_{197} -specific Ab comparable to those elicited by 6B- and 19F-CRM_{197} . All three conjugates also elicited comparable CRM_{197} -specific T cell proliferative responses. We now report that BALB/c and (BALB/c x CBA/J)F_1 mice produce significantly higher titers of 23F-specific antibodies after immunization with 23F- CRM_{197} than do CBA/J mice. There were significantly fewer 23F-specific antibody-secreting cells in CBA/J mice as compared to BALB/c or F_1 mice ($p<.001$). The decreased ability of CBA/J mice to respond to 23F PnPS may be due to a genetically-influenced decrease in the frequency of 23F-specific B cell precursors as compared to BALB/c and (BALB/c x CBA/J)F_1 mice.

42.16

A DNA CONSTRUCT ENCODING HSV-1 GLYCOPROTEIN D DECREASES VIRAL REPLICATION IN THE CNS OF MICE.

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This study explores the antiviral effects of a DNA vaccine against HSV-1 glycoprotein D (gD). The nuclease-insensitive construct (gD-ASOR) consists of an HSV-1 gD encoding plasmid coupled to asiolorosomucoid (ASOR), which targets it to cells bearing ASOR receptors. In BALB/c mice, the construct generates a CD4$^+$ cytotoxic T cell response against HSV-1, while in C3H mice it triggers a CD8$^+$ cytotoxic T cell response against HSV-1. Since a CD4$^+$ T cell response is critical in controlling recurrent HSV-1 infections in humans, the response of BALB/c mice will have greater clinical relevance. Mice were immunized with two 10 μg doses of gD-ASOR spaced seven days apart. Fourteen days after the second immunization mice were infected by the corneal route with 10^6 pfu HSV-1, strain 17syn+. Acute replication kinetics and flow cytometry were carried out on days 2, 4, 6, and 10 post infection. By day 6, there was a 100-fold decrease in viral titers in the trigeminal ganglia from immunized BALB/c mice compared to sham-immunized mice. Viral titers in the trigeminal ganglia from immunized C3H mice were 6-fold lower than sham-immunized mice. A decrease in the magnitude of viral replication in the trigeminal ganglia from immunized mice suggests that our construct limits the establishment of latent HSV-1 infection. (Supported by NIH Grant #1-R03 DE12521-01A1, Immune Response Corporation, and Wright State University BMS PhD Program)

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RESEARCH ARTICLE

Low dose and gene gun immunization with a hepatitis C virus nonstructural (NS) 3 DNA-based vaccine containing NS4A inhibit NS3/4A-expressing tumors in vivo

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The hepatitis C virus (HCV) protease and helicase encompasses the nonstructural (NS) 3 protein and the cofactor NS4A, which targets the NS3/4A-complex to intracellular membranes. We here evaluate the importance of NS4A in NS3-based genetic immunogens. A full-length genotype 1 NS3/4A gene was cloned into a eucaryotic expression vector in the form of NS3/4A and NS3 alone. Transient transfections revealed that the inclusion of NS4A increased the expression levels of NS3. Subsequently, immunization with the NS3/4A gene primed 10- to 100-fold higher levels of NS3-specific antibodies as compared to immunization with the NS3 gene. Humoral responses primed by the NS3/4A gene had a higher IgG2a/IgG1 ratio (>20) as compared to the NS3 gene (3.0), suggesting a T helper 1-skewed response. Low dose i.m. (10 μg) immunization with the NS3/4A gene inhibited the growth of NS3/4A-expressing tumor cells in vivo, *whereas the NS3 gene alone or NS3 protein did not. We then evaluated the efficiency of the NS3/4A gene administered by the gene gun, at the same doses used for humans, in priming cytotoxic T lymphocyte (CTL) responses. Three to four 4 μg doses of the NS3/4A gene primed CTL at a precursor frequency of 2–4%, which inhibited the growth of NS3/4A-expressing tumor cells in vivo. Thus, NS4A enhances the expression levels and immunogenicity of NS3, and an NS3/4A gene delivered transdermally could be a therapeutic vaccine candidate.*

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Keywords: *hepatitis C virus; HCV; NS3; DNA vaccine; gene gun*

Introduction

Most patients infected with the hepatitis C virus (HCV) develop chronic infections. Although the mechanism responsible for viral persistence is still largely unknown, the high variability of HCV is widely believed to play an important role.[1] The genetic heterogeneity of HCV is a result of the high viral replication rate of 10^{10}–10^{13} viral particles daily[2] together with spontaneous nucleotide substitutions of 10^{-2}–10^{-3} substitutions per nucleotide per year.[3,4] Thus, the viral variability severely complicates vaccine development.

Vigorous and multispecific CD4-mediated responses directed against structural and nonstructural (NS) HCV antigens are present in the acute phase of HCV infection of patients who resolve the infection.[5–7] In contrast, these responses are significantly weaker, or even absent, among patients with acute HCV infection who progress to chronicity.[5–7] These data suggest that the intensity of the T-cell reactivity at the early stages of infection may be critical to limit the spread of the virus within the infected host, and to keep viral replication under control.

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Vaccine development should, based on the above, aim at inducing T cells directed to genetically stable regions of the viral genome. It has been shown that DNA immunizations can induce both specific antibodies and cell-mediated responses against the structural and nonstructural HCV proteins in mice.[8–18] One attractive region for vaccine development is the NS3 protein. However, when compared to responses to other, more genetically unstable, regions of the genome, the NS3-specific responses do not seem to be easily primed. In most studies poor humoral responses have been reported.[9,10,13] In addition, only a few studies have suggested the priming of potent cell-mediated responses.[9,15] We recently noted that when using a genetic immunogen containing the complete NS3/4A protease, the humoral responses were surprisingly strong.[15] The reason for this was not clear, although different reports suggest that the presence of the cofactor NS4A increases the intracellular stability of NS3.[19,20] This may be explained by the fact that the amino terminal domain of NS4A targets the NS3/4A complex to intracellular membranes.[20] In fact, both the protease and helicase activities of HCV NS3 require the presence of NS4A.[21–24] We have now found evidence that NS4A is of vital importance also for the immunogenicity of HCV NS3 when used as a genetic immunogen.

Results

Characterization of NS3 and NS3/4A expression constructs

The expression constructs NS3-pVAX1 and NS3/4A-pVAX1 were analyzed by polymerase chain reaction (PCR) to verify the size of the amplicons. All vectors were sequenced to ensure the integrity of the gene. The expression of NS3, NS3/4A and mNS3/4A by the plasmid was analyzed by an *in vitro* transcription and translation assay. The assay showed that the proteins could be correctly translated from the plasmids (Figure 1). We have observed previously that two bands become visible after *in vitro* translation of the NS3/4A plasmid.[15,25] This suggests that the cleavage between NS3 and NS4A mediated by the NS3 protease may not be complete in the *in vitro* translation assay. By introducing a targeted mutation that replaces the P1′ serine with a proline at the NS3/4A proteolytic site,[26,27] only the band representing the expected NS3/4A fusion protein became visible (Figure 1). Collectively, the NS3 and NS3/4A plasmids express the full-length genes and the protease activity of NS3 is intact.

The expression levels obtained with the NS3, NS3/4A and mNS3/4A genes were compared using a Semliki forest virus (SFV) vector based expression system. Nonproductive infection of BHK cells with SFV vectors expressing the three genes revealed that the NS3/4A gene with an intact proteolytic site gave the highest expression of NS3 (Figure 2). Thus, the inclusion of NS4A improves the expression of NS3. The reason for this effect needs to be determined. Also, staining of rSFV-NS3- and rNS3/4A-infected BHK cells revealed a slightly different intracellular distribution of NS3 (Figure 2). The NS3 protein expressed by infection with rSFV-NS3 displayed a more diffuse staining pattern as compared to rSFV-NS3/4A at 24 h postinfection, possibly indicating the membrane targeting conferred by NS4A. This needs to be further explored.

Comparison of humoral responses following DNA immunization with NS3 and NS3/4A

To test the immunogenicity of different NS3 genes, groups of BALB/c (H-2^d) mice were immunized with recombinant (r) NS3, and the NS3, NS3/4A and mNS3/4A genes. The mice were boosted every fourth week. BALB/c mice were used since they have been shown to be good responders to NS3 but low/nonresponders to NS4A of genotype 1.[15,28,29] Thus, any differences in the immune response cannot be attributed to the addition of new CD4+ T helper (Th) epitopes.

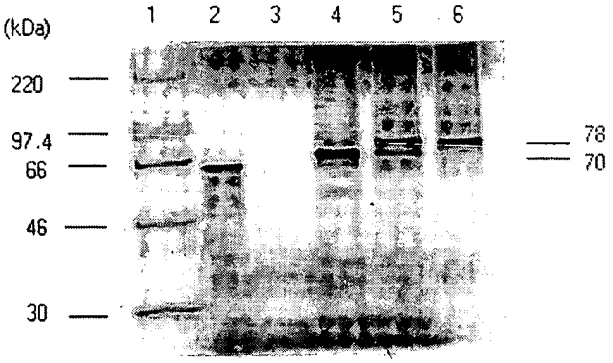


Figure 1 Analysis of the translation products from the plasmids NS3-pVAX1, NS3/4A-pVAX1, and mNS3/4A-pVAX1 by in vitro translation in the presence of ^{35}S-methionine and SDS-PAGE electrophoresis. Lane 1, molecular weight marker (CFA 756; Amersham Pharmacia Biotech); lane 2, 61 kDa kit control; lane 3, negative control; lane 4, NS3-pVAX1; lane 5, NS3/4A-pVAX1; and lane 6, mNS3/4A-pVAX1.

To compare directly the immunogenicity of NS3 and NS3/4A genes, two groups of five H-2^d mice each were immunized with 100 μg NS3-pVAX1 or NS3/4A-pVAX1. The mice immunized with NS3/4A-pVAX1 had a more rapid antibody response, suggesting that the NS3/4A plasmid had a higher intrinsic immunogenicity (Figure 3). Even after four immunizations the mice immunized with NS3/4A had higher antibody levels (Figure 3). In order to further confirm this observation and to explain the importance of NS4A, larger groups of mice were immunized with NS3-pVAX1 only expressing NS3, NS3/4A-pVAX1 expressing both NS3 and NS4A, and the mutant NS3/4A plasmid only expressing the NS3/4A fusion protein. The differences in immunogenicity between NS3-pVAX1 and NS3/4A-pVAX1 plasmids were perfectly reiterated (Figure 3). The NS3/4A gene was more immunogenic than the NS3 gene alone with respect to mean antibody levels and the frequency of responding mice (Figure 3). Interestingly, in the early immune response; that is at 2 and 4 weeks, the NS3/4A-pVAX1 plasmid was also more immunogenic than the mNS3/4A-pVAX1 plasmid (Figure 3). Thus, to utilize fully the benefit of NS4A in the plasmid, a functional proteolytic site between NS3 and NS4A seems to be of importance. This is fully consistent with the previously noted effect of NS4A on the expression levels of NS3.

To test the possibility as to whether a new Th epitope, generated at the junction of the NS3 and NS4A proteins, was responsible for the increased immunogenicity seen with the NS3/4A gene, T cell proliferation assays were performed. BALB/c mice were immunized with rNS3 or NS3/4A-pVAX1 and 9 days later the spleen cell recall cultures set in *in vivo* primed cells were recalled for 5 days with rNS3 and a 20 amino acid (aa) peptide spanning the NS3/4A junction. As shown in Figure 3, both rNS3 and NS3/4A-pVAX1 primed T cells were recalled *in vitro* by rNS3. Neither rNS3 or NS3/4A-pVAX1 primed T cells could be recalled by the NS3/4A junctional peptide. The same results were reiterated in C57BL/6 (H-2^b) mice (data not shown). Thus, no new T helper cell site had been generated by the NS3/4A fusion protein.

Comparison of T helper (Th) cell responses following DNA immunization with NS3 and NS3/4A

To compare the proliferative Th cell responses between NS3 and NS3/4A, groups of mice were immunized with 100 μg of plasmid, and 13 days later spleen cells were harvested and *in vitro* recall assays were set using rNS3. The level of NS3-specific Th cell priming was more efficient in the NS3/4A-immunized mice as compared to the NS3-immunized mice (Figure 4). Both the level of T-cell proliferation was higher and the amount of rNS3 required *in vitro* was lower to recall a detectable response.

The Th-cell phenotype primed by NS3/4A immunization has been described in detail previously.[15] To

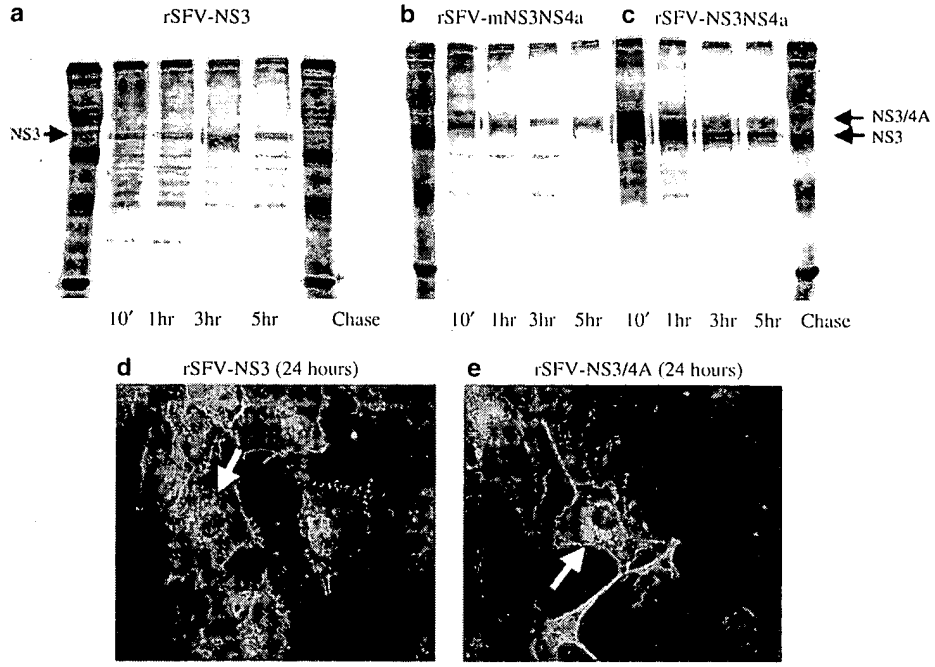


Figure 2 Analysis of NS3 expressed by rSFV-NS3 (a), mNS3/4a (b), or NS3/4a (c) infected BHK-21 cells. After labelling with [^{35}S] methionine, cells were 'chased' with cold methionine for the indicated times. The resulting cell lysates were analyzed by immunoprecipitation and 10% SDS-PAGE. NS3 expression was further demonstrated in rSFV-NS3 (d) and rSFV-NS3/4A (e) infected BHK cells, by immunofluorescent staining, using an NS3-specific monoclonal antibody. Cells stained 24 h after infection suggest a greater dispersion of the NS3 protein in rSFV-NS3 infected cells (e).

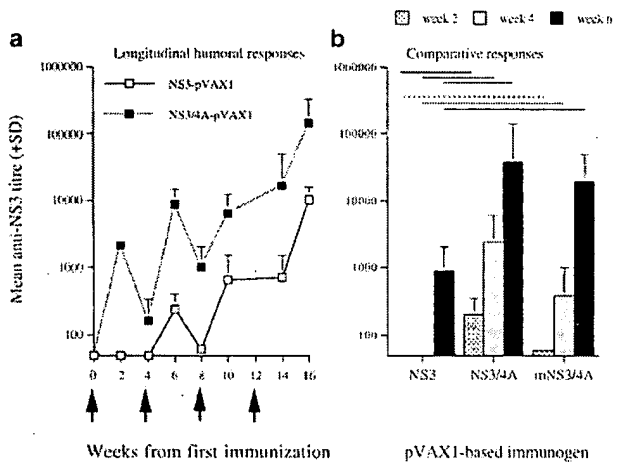


Figure 3 Antibody responses primed by immunizations with 100 μg NS3-pVAX1 or NS3/4A-pVAX1 in groups of five H-2^d mice (a). Arrows indicate time point of immunization. All mice were pretreated with cardiotoxin. Values are given as mean end-point antibody ±s.d. Also shown are a comparison of the humoral responses primed by 100 μg NS3-pVAX1, NS3/4A-pVAX1, or mNS3/4A-pVAX1 in groups of 10–20 H-2^d mice. Mice were primed and boosted at week 0 and 4. Values are given as mean end-point antibody titre ±s.d. A solid line indicates a significant difference of $P<0.01$, a broken line a difference of $P<0.05$, and a dotted line no significant difference (Mann–Whitney U-test).

compare directly the T helper 1 (Th1) and Th2 skewing of the T-cell response primed by NS3 and NS3/4A immunization, the levels of NS3-specific IgG1 (Th2) and IgG2a (Th1) antibodies were analyzed (Figure 4). In H-2^d and H-2^k mice immunized with rNS3 in PBS or adjuvant, IgG1 was the dominant subclass. The IgG2a/IgG1 ratio in mice immunized with rNS3 was always <1 regardless of the murine haplotype,[28] which signals a Th2-dominated response.[30] In contrast, immunized NS3-pVAX1 or NS3/4A-pVAX1 mice had Th1-skewed Th-cell responses evidenced by IgG1/IgG2a ratios of >1. However, the subclass ratio in NS3-pVAX1-immunized mice suggested a mixed Th1/Th2 response (Figure 4). In contrast, none of the NS3/4A-pVAX1-immunized mice had IgG1, indicating a profoundly Th1-skewed response. Thus, the priming of Th- cells is also influenced by the presence of NS4A (Figure 4).

In vivo protection against growth of NS3/4A-expressing tumor cells

An efficient way to determine *in vivo* functional immune responses is the determination of inhibition of tumor growth *in vivo* in BALB/c mice using SP2/0 myeloma cells expressing the desired viral antigen.[10] The inhibition of tumor growth following DNA immunization was shown to be fully dependent on an efficient priming of specific CTLs.[10] One could argue that CTL responses to viruses differ as compared to the responses to tumor cells. However, this model does offer some advantages as compared to, for example, recombinant vaccinia viruses, since no irrelevant viral proteins (ie vector-derived) are produced by the cell. Thus, this should therefore be an adequate model to study *in vivo* functional immune responses to NS3/4A. An SP2/0 cell line stably expressing NS3/4A was therefore made. The *in vivo* growth kinetics of the NS3/4A-expressing cell line was fully comparable to the parental cell line (data not shown).

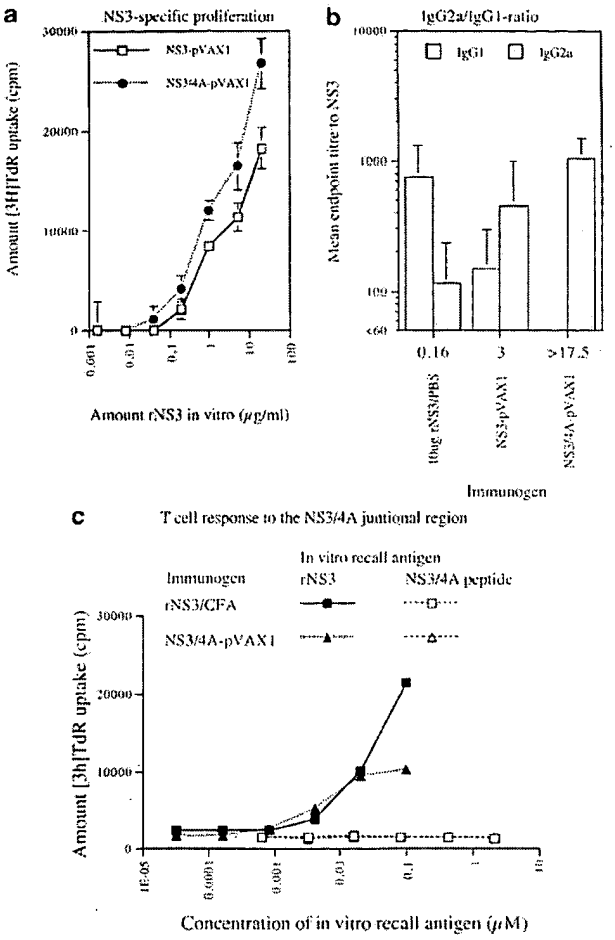


Figure 4 *T-cell responses to NS3 in spleens from immunized H-2^d mice. Groups of five mice were immunized with 100 μg NS3-pVAX1 or NS3/4A-pVAX1. All mice were pretreated with cardiotoxin. Values are given as the antigen-induced proliferation minus the spontaneous proliferation (Δcpm). Values are shown as mean cpm values ±s.d. of triplicate determinations (a). Comparison of the NS3-specific IgG subclass response at week six in BALB/c mice immunized with rNS3 (10 μg) in PBS, NS3-pVAX1 or NS3/4A-pVAX1 (b). Values have been given as the mean end point titre ±s.d. of IgG1 or IgG2a antibodies to NS3. The titer ratios were obtained by dividing the mean endpont titer of IgG2a antibodies to NS3 by the mean endpont titer IgG1 antibodies to NS3. A high ratio (>3) indicates a Th1-like response and a low ratio (<0.3) indicates a Th2-like response, whereas values within a three-fold difference from 1 (0.3–3) indicates a mixed Th1/Th2 response. Also given (c) are the proliferative responses in the spleen after one immunization with rNS3 in CFA, after 3 monthly injections with the NS3/4A-pVAX1 plasmid given i.m. (these mice were killed 6 weeks after the last injection). Values are shown as mean cpm values of triplicate determinations (c).*

We first determined how many DNA injections were needed to prime CTLs that lysed the NS3/4A-expressing cells *in vitro*. Mice were pretreated with cardiotoxin and were given 2, 3, or 6 monthly injections in TA muscles of 100 μg NS3/4A-pVAX1. Groups of five mice were killed 2 weeks after each injection. We found that 3–6 intra muscular (i.m.) injections were needed for the priming of *in vitro* detectable CTLs (Figure 5).

To ensure that *in vivo* active CTLs were primed, all mice received five immunizations prior to *in vivo* challenge with the NS3/4A-expressing cells. There was no difference in tumor growth among groups of mice immunized with PBS, or with a control plasmid expressing the p17 protein of human immunodeficiency virus type 1 (Iroegbu *et al*[31]; Figure 6). In addition, mice immunized with rNS3 in CFA did not show inhibition of tumor growth, confirming that the priming of specific B and Th cells alone does not confer tumor protection in this model (Figure 6). In contrast, immunization with 100 μg of NS3-pVAX1 or NS3/4A-pVAX1 significantly reduced tumor growth at all time points (Figure 6). Interestingly, immunization with mNS3/4A showed significant inhibition of tumor growth at days 7 and 13, but not at day 11. By reducing the dose of plasmid 10-fold, the ability to prime inhibiting responses was lost for the NS3-pVAX1 plasmid, but not for the NS3/4A-pVAX1 plasmid (Figure 6). Thus, NS4 enhances the immunogenicity of NS3 also in the priming of *in vivo* tumor protecting immune responses. Interestingly, the presence of a functional cleavage site at the NS3/4A junction seems to be important to utilize fully the benefits of NS4A. Importantly, the slightly lower protection conferred by immunization with mNS3/4A-pVAX1 expressing the NS3/4A fusion protein (Figure 6) shows that the importance of NS4A cannot be explained by new epitopes carried by NS4A or the NS3/4A junctional region.

Despite immunization, most mice developed tumors, albeit at a lower growth rate. To study the development of tumors in the different experimental groups, sections were prepared from all harvested tumors. In tumors developing in mock-immunized mice, necrosis was a common occurrence, with central cell death characterized by the presence of pycnotic nuclear remnant (Figure 7). In corresponding sections stained for CD3 antigen, only a sparse infiltration of positive T lymphocytes was noted (Figure 7). A similar picture was seen in mice immunized with recombinant NS3 (data not shown). In DNA-immunized animals occasional necrotic areas could be seen. However, large tumor areas had been replaced by oedematous and vascularized tissue (Figure 7). These areas were densely infiltrated by CD3 positive lymphocytes. At the interface to viable tumor tissue, an accumulation of lymphocytes was noted as well as apoptotic cells, probably representing dying myeloma cells (Figure 7). In addition, staining by the CD3 antibody revealed a major invasion of T cells in areas with tumor regression (Figure 7). Thus, this further confirms that T cells, presumably CTLs, are responsible for the observed inhibition of tumor growth.

Evaluation of the NS3/4A-pVAX1 plasmid administered by gene gun immunization

All data obtained strongly suggest that the NS3/4A-based DNA immunogen has the properties desired in a therapeutic vaccine for chronic HCV infection. Although injections in regenerating muscle tissue are effective for DNA immunizations in mice, such treatments are unrealistic for human use. We therefore evaluated the performance of the NS3/4A-pVAX1 immunogen by transdermal delivery using the gene gun, which is presently evaluated in humans. To evaluate the efficiency of transdermal plasmid administraion in priming CTLs, we first had to develop the reagents needed to quantify the CTL responses by flow cytometry.

To quantify NS3/4A-specific CTLs using a divalent MHC:Ig fusion protein,[32] we first identified a peptide corresponding to an H-2^b-restricted NS3-specific CTL epitope. NS3/4A-specific CTL epitopes were identified from a set of overlapping 20 aa synthetic peptides spanning NS3/4A. The 20 aa peptides were assayed for the stabilization of surface expression of MHC class I molecules on transporter associated with antigen processing (TAP) 2 deficient RMA-S cell line.[33,34] By this assay, one peptide was identified that bound H-$2D^b$ molecules with high affinity (data not shown). To verify the optimal peptide sequence, nine aa long peptides, with an eight aa overlap, were synthesized and evaluated for H-$2D^b$ binding (data not shown). This identified one candidate peptide (sequence GAVQNEVTL), located at the C-terminal domain of NS3, 21 aa from the NS3/4A junction. The peptide was then used to immunize C57BL/6 (H-2^b) mice. Splenocytes from immunized mice were harvested, restimulation cultures were set with the NS3 peptide and irrelevant peptides, and 5 days later the effector cells were tested for lysis of peptide-loaded RMA-S cells. NS3/4A-specific CTLs could only be detected in splenocytes from peptide-immunized mice that had been restimulated with the NS3/4A peptide (Figure 8). To test whether the NS3-derived CTL peptide could be recognized by CTLs primed by NS3/4A-pVAX1 immunization using gene gun, spleens from DNA-immunized mice were restimulated with the NS3 peptide and evaluated for lysis of peptide-loaded RMA-S cells. These experiments showed that mice immunized transdermally with NS3/4A-pVAX1 using the gene gun developed NS3-specific CTLs only when splenocytes had been restimulated with the NS3 peptide and not an irrelevant peptide (Figure 8).

We could now quantify specific CTLs directly *ex vivo*. The advantage of this technique is that it bypasses the possible disadvantages of *in vitro* expansion of CTLs prior to analysis. Direct *ex vivo* quantification of NS3-specific CTLs using NS3-peptide-loaded divalent H-$2D^b$:Ig fusion protein molecules revealed that around 2–4% of the CD8+ population in the spleens from mice immunized transdermally with NS3/4A-pVAX1 using the gene gun were specific for NS3/4A (Figure 9). This is fully consistent with the effective lysis of peptide-loaded cells recorded in the lytic assays. Thus, NS3/4A-pVAX1 effectively primes a large population of specific CTLs that are readily detectable *in vitro* and that recognize a fine mapped H-$2D^b$ binding NS3-specific CTL epitope.

Evaluation of the in vivo priming of tumor-inhibiting immune responses by gene gun immunization

To test the efficiency of the *in vivo* primed NS3/4A-specific CTL responses following transdermal administration, immunized mice were challenged with the NS3/4A-expressing SP2/0 tumor cell line. Previous experiments had shown that four transdermal injections primed a high precursor frequency of NS3/4A-specific CTLs. Groups of 10 BALB/c mice were either left untreated or given four injections with 4 μg of the NS3/4A-pVAX1 plasmid at monthly intervals. A total dose of 16 μg NS3/4A-pVAX1 plasmid effectively primed CTL responses *in vivo* that significantly inhibited tumor growth (Figure 10). Thus, using the same route and dose as previously used in humans,[35] we found that the NS3/4A-pVAX1 plasmid primed tumor-inhibiting immune responses. This strongly suggest that the NS3/4A-pVAX1 plasmid delivered transdermally may be a viable approach also in humans with the aim of priming HCV-specific CTLs.

Discussion

Several vaccine approaches are being tested for HCV infections. Many of these rely on genetically variable regions of the virus, such as the envelope (e) 1 or e2 proteins. A problem with vaccines based on viral genes with a high degree of variability is that the immune

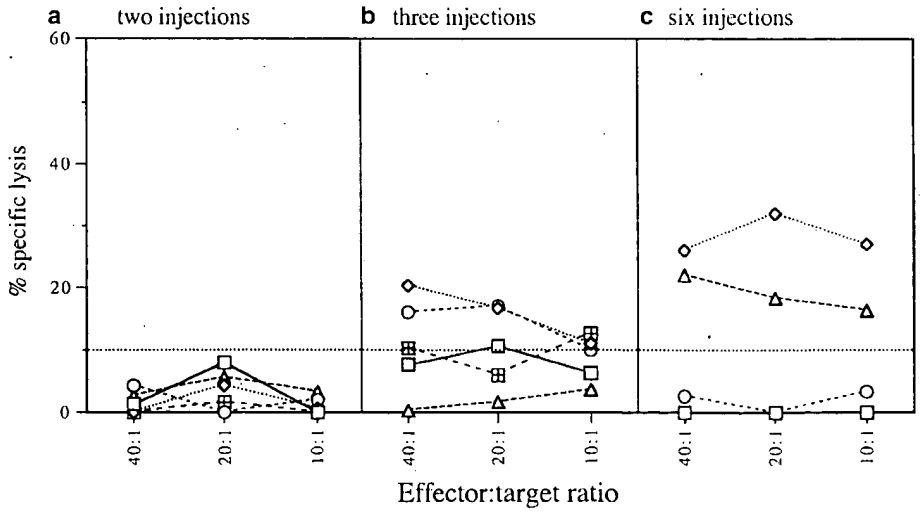
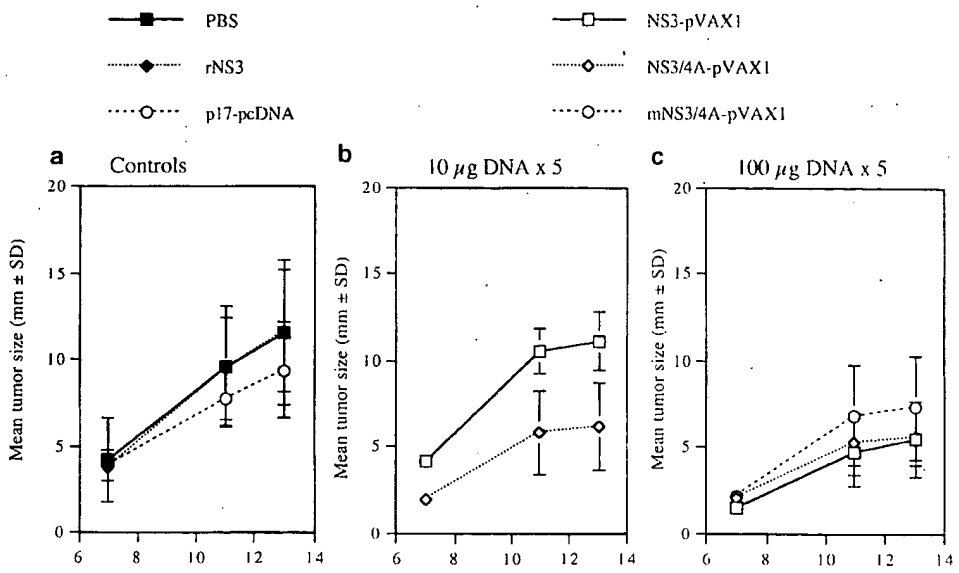


Figure 5 Kinetics of the priming of in vitro detectable CTLs in H-2^d mice. Groups of five H-2^d mice were immunized i.m. with 100 μg NS3/4A-pVAX1 at monthly intervals. All mice were pretreated with cardiotoxin. Results from the cytotoxicity assays have been given from two injections (a), three injections (b), and six injections of 100 μg DNA (c). The percent specific lysis corresponds to the percent lysis obtained with NS3/4A expressing SP2/0 cells minus the percent lysis obtained with nontransfected SP2/0 cells. Values have been given for effector to target (E:T) cell ratios of 40:1, 20:1 and 10:1. More than 10% specific lysis was considered as positive. Each line indicate an individual mouse.



d Statistical comparizon of AUC values

Group	NS3-pVAX1		NS3/4A-pVAX1		mNS3/4A-pVAX1
	10μg	100μg	10μg	100μg	
PBS	NS	p<0.01	p<0.01	p<0.01	p<0.01
rNS3	NS	p<0.01	p<0.01	p<0.01	p<0.01
p17-pcDNA3	NS	p<0.05	NS	p<0.05	NS

Figure 6 Inhibition of in vivo tumor cell growth by different modes of immunization. Groups of five to 10 H-2^d mice were immunized with either PBS or 20 μg rNS3 in CFA given intra peritoneally or 100 μg of control plasmid (p17-pcDNA3) (a) or with 10 μg of NS3-pVAX1 or NS3/4A-pVAX1 (b) or 100 μg of NS3-pVAX1 or NS3/4A-pVAX1 or mNS3/4A-pVAX1 (c). Mice were primed and boosted at week 4, 8, 12 and 16. All mice were pretreated with cardiotoxin. Mice were injected with 2 x 10^6 NS3/4A-expressing SP2/0 cells s.c. 2 week after last immunization. Tumor sizes were measured through the skin at days 7, 11 and 13 after tumor injection. Mean tumor growth in each group was for the whole period and groups were compared statistically using area under the curve (AUC) and ANOVA. (d) shows statistical comparisons between the experimental groups and the control groups have been given.

responses primed by the vaccine may not recognize the virus of the infected host. Thus, there are ample arguments as to why vaccines against viruses with a high genetic diversity should be based on the most genetically stable regions of the virus. In the HCV genome, two regions are well conserved between different strains, the core and NS3 regions. Unfortunately, neither of these has been found to be highly immunogenic in animal models.[10,14,16] Several factors favor the use of NS3. First, NS3 is a large protein whereby a genetic nonresponder status on the T-cell level is unlikely, as previously suggested from murine experiments.[28] Second, the reason for the genetic stability is most likely the three different enzymatic properties contained within NS3, protease, helicase and NTPase. Thus, many genetic changes within NS3 will affect the viral replication. Third, numerous studies have now found that NS3-specific T-cell responses correlate with resolution of the infection.[5–7]

We noted early on that our DNA-based immunogen comprising the complete protease of HCV that is, both NS3 and NS4A, induced much higher antibody levels than previously reported.[10,13] This suggested that NS4A might affect the immunogenicity of NS3. It should be noted that NS4A as a cofactor for the NS3 protease has the ability to target the NS3/4A complex to intracellular membranes, and to prolong the intracellular half-life of NS3.[19,20] Taken together, these data indeed suggest that the 54 aa NS4A protein may also affect the immunogenicity of NS3. The present study was undertaken to resolve this issue.

To better understand why NS4A is important for the immunogenicity of NS3, we designed mutant versions of the NS3/4A gene. It is presumed that NS3 cleaves the NS3/4A junction in trans and that the free NS4A binds to the NS3 protease domain.[36,37] Using *in vitro* translation assays, we found that the NS3/4A gene gave rise to two bands around 70–78 kDa. The smaller band was presumed to be NS3 and the larger band was presumed to be the uncleaved NS3/4A fusion protein. By replacing the junctional Thr-Ser-Thr motif by a Thr-Pro-Thr motif, it became clear that the proteolytic site had been

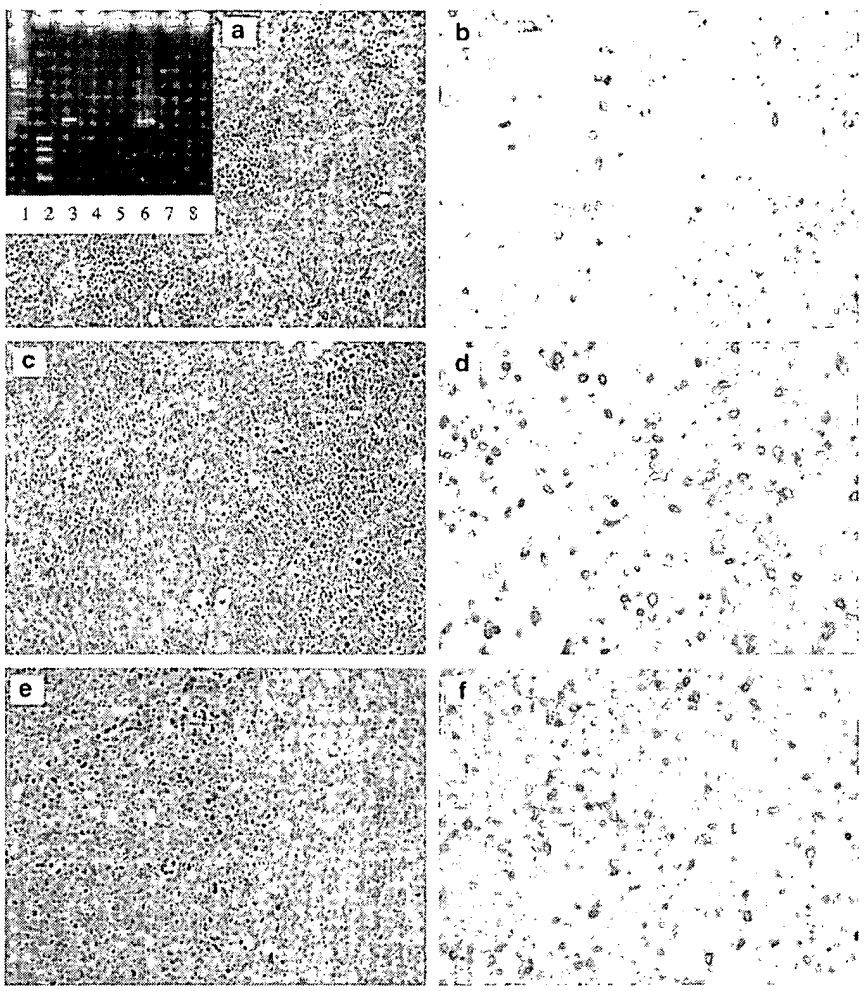


Figure 7 *Histological appearance of solid tumors excised from nonimmunized mice (a and b), mice immunized with 10 μg NS3/4A-pVAX1 (c and d), and mice immunized with 100 μg NS3/4A-pVAX1 (e and f). Sections of NS3/4A expressing SP2/0 myeloma stained by hematoxylin–eosin (a, c, and e) or by anti-CD3 antibody (b, d, and f). The inset in (a) show the results from testing the transfected cell line for expression of NS3/4A mRNA by RT-PCR. Lanes 1 and 2 shows the molecular weight markers, lane 3 the NS3/4A-SP2/0 cells, lane 4 the SP2/0 cells, lanes 5, 7, and 8 are negative controls, and line 6 a DNA PCR of the NS3/4A-pVAX1 plasmid giving a band of 2061 bases.*

destroyed since only the NS3/4A fusion protein could be detected as a translation product from the mutant plasmid. Interestingly, the mutant construct primed an intermediate humoral response as compared to the NS3 and NS3/4A genes. Also, the mutant NS3/4A gene was less efficient in priming tumor-inhibiting responses *in vivo*. This suggests that new NS3/4A junctional epitopes cannot explain the beneficial effects of NS4A, and thus a functional cleavage site between NS3/NS4A seems to be needed to utilize fully the effects of NS4A. By comparing the expression levels of the NS3, NS3/4A and the mNS3/4A genes, in transient transfections we found that the presence of NS4A in its native form increased the expression levels of NS3.

The inclusion of NS4A in the NS3-based DNA immunogen had several beneficial effects. The NS3-specific humoral responses appeared quicker and reached higher titers, which show that the intrinsic immunogenicity of the NS3 protein had been improved. In addition, the priming of Th cells was more effective and the Th1/Th2 balance was shifted towards Th1. Finally, an *in vivo* CTL-dependent inhibition of NS3/4A-expressing tumor cells could be obtained at 10-fold lower doses of the immunogen when NS4A was present. Thus, the presence of NS4A in the context of a heterodimer or a fusion protein with NS3 has superior immunogenic properties as compared to NS3 alone. What could be the possible explanations for this observation? First, we did find that the expression levels of NS3 were improved by the inclusion of NS4A. This finding alone can most likely explain the effects on immunogenicity. Second, it has been shown that the presence of NS4A increased the intracellular survival of NS3. This would presumably also add to the observed immunogenic characteristics of the NS3/4A gene. Alternative explanations could be that the NS3/4A-pVAX1 plasmid itself has some immune stimulating properties or that a new T-cell site has been generated. We could show that a control DNA sequence (CpG-1826; Hartman *et al*[38]) did activate B cells (data not shown). However, comparing the activation of B cells by the addition of NS3- and NS3/4A-pVAX1 plasmids, no difference as determined by flow cytometry could be

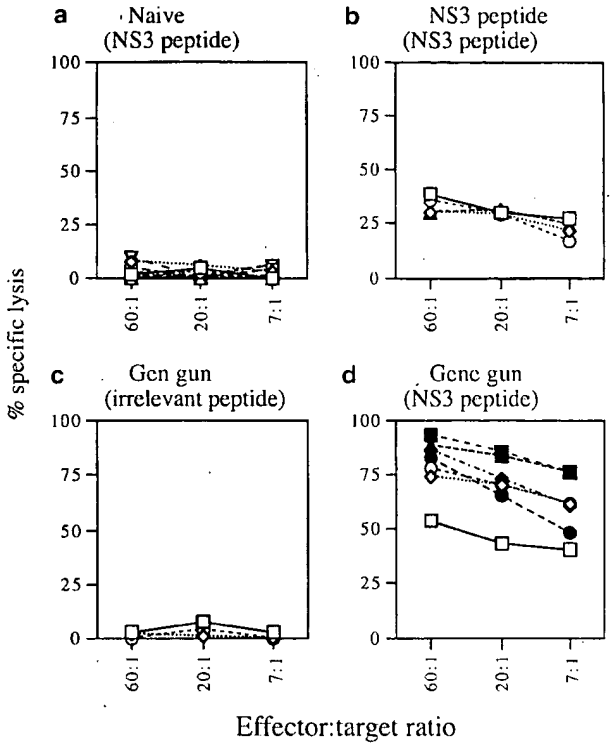


Figure 8 Gene gun immunization with NS3/4A-pVAX1 induces CTL specific for an H-2D^b–restricted peptide epitope. Groups of five to ten C57BL/6 mice were immunized s.c. with 100 μg NS3-specific peptide (GAVQNEVTL) in CFA or transdermally with 4 μg DNA/dose using the gene gun at monthly intervals. Spleen cells from naive (a) or NS3 peptide-immunized mice (b) or NS3/4A-pVAX1 gene gun-immunized mice (d) were restimulated 5 days in vitro with irradiated NS3-peptide loaded naive spleen cells. Spleen cells from gene gun-immunized mice restimulated with an irrelevant H-2D^b binding peptide served as negative control (c). In panel (d) white boxes indicates the % specific lysis after three immunizations and black boxes represent the % specific lysis after four immunizations. Within the parentheses the peptide used in the restimulation cultures have been indicated. Each line represents data from an individual mouse.

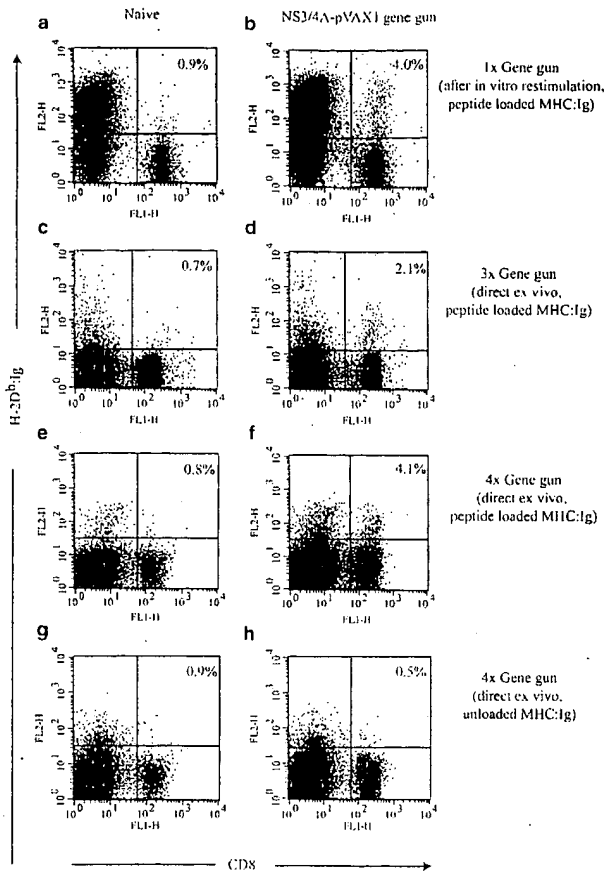


Figure 9 Induction of NS3/4A-specific CD8 T cells after gene gun immunization. The frequency of NS3 peptide-specific CD8 T cells were determined by flow cytometric staining of spleen cells from nave mice (a, c, e, and g) and NS3/4A-pVAX1 DNA-immunized mice (b, d, f and h) with dimeric H-2D^b:Ig fusion protein loaded with the NS3 peptide (GAVQ-NEVTL). Unloaded H-2D^b:Ig fusion protein was used to monitor unspecific staining (g and h). A total of 150 000–200 000 cells were collected and the percentage of CD8+ cells stained for H-2D^b:Ig are indicated in the parentheses in each dot-plot.

observed (data not shown). Also, no junctional T-cell reactivity could be detected. Hence, we favor the hypothesis that the increased immunogenicity of NS3 by the inclusion of NS4A is, at least in part, explained by an increased expression of NS3.

Today there are many arguments that one major aim of a therapeutic vaccine is to prime virus-specific CTL. In particular when a nonstructural viral protein is used, CTLs may be a crucial component of the antiviral response. We could show that NS3-specific CTLs are effectively primed by the NS3/4A-pVAX1 plasmid administered i.m. or transdermally. Importantly, the identification of an H-2D^b-restricted NS3/4A-derived CTL epitope allowed for a direct *ex vivo* quantitation of the CTL response after immunization. Four gene gun immunizations with 4 μg plasmid per dose elicited NS3 specific T cells that corresponded to approximately 4% of the whole splenic CD8+ population. In addition, these responses were active *in vivo* and inhibited growth of NS3/4A-expressing tumor cells. Thus, the NS3/4A-pVAX1 immunogen when administered transdermally at low doses is highly effective in priming NS3-specific CTLs, one of the major goals with therapeutic vaccine regimens. In fact, this is the same DNA dose as already used in human vaccine trials.[35]

In conclusion, we have found that the inclusion of NS4A in NS3-based DNA immunogens is essential for the immunogenicity of NS3, presumably due to the observed increased expression levels of NS3 conferred by NS4A *in vitro*. All parameters of the NS3-specific immune response were improved by the addition of NS4A. We could show that this is not explained by the generation of a new T-cell site at the NS3/4A junction. Additionally, the mice used (H-2^d) do not respond to NS4A of genotype 1,[29] and in T cell recall experiments only NS3 was used as the recall antigen. Consistent with this the mutant NS3/4A gene did not show increased expression levels, and was not as effective as the native NS3/4A gene in priming CTL responses *in vivo*. Finally, the 150-bp NS4A sequence does not add any immune-stimulating motifs, such as the CpG motif, and the NS3/4A-pVAX1 plasmid did not activate B cells any differently than the NS3-containing plasmid. Thus, all available data suggest that the increased immunogenicity of

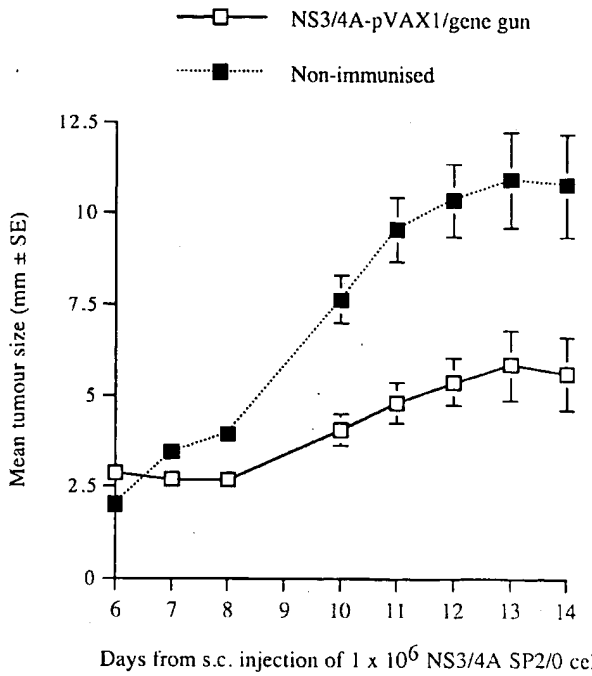


Figure 10 *Inhibition of in vivo tumor growth by gene gun immunization. Groups of ten BALB/c mice were either left untreated or were given 4 monthly transdermal immunizations with 4 μg DNA/dose of NS3/4A-pVAX1. Mice were injected s.c with 1×10^6 NS3/4A-expressing SP2/0 cells 4 weeks after last immunization. Tumor sizes were measured through the skin at days 6, 7, 8, 10, 11, 12, 13, and 14, 15 after tumor injection. The area under the curve for the two curves was statistically different (ANOVA; $P < 0.01$).*

NS3 by the inclusion of NS4A can only be explained by the observed increase in expression levels obtained by the inclusion of NS4A. The importance of NS4A in the immunogenicity of NS3 is, thus, most likely explained by the fact that NS4A somehow affects the expression levels of NS3, either as the NS3/4A heterodimer or by NS4A alone. Regardless of the mechanism, the increased expression of NS3 observed *in vitro* by the inclusion of NS4A is fully consistent with the improved immunogenic properties observed using the NS3/4A gene. However, further experiments are needed to resolve the mechanism behind the increased expression levels.

Importantly, the NS3/4A gene effectively primes a high precursor frequency of CTLs that inhibit the growth of NS3/4A-expressing tumor cells *in vivo* when administered transdermally, a delivery route thought mainly to prime Th2-like immune responses.[30,39–41] Thus, the NS3/4A gene delivered transdermally has many attractive features desired in a therapeutic vaccine.

It is generally believed that a therapeutic vaccination schedule, where the antigens are delivered under optimal immunigenic conditions, may improve the immune responses against the virus infecting the host despite the constant presence of viral proteins. This approach has been shown to be effective in animals infected with the hepatitis B virus.[42,43]

With respect to chronic HCV infections, there are some reasons as to why therapeutic vaccination could help existing therapies. A previous infection with HCV confers partial protection against reinfection, suggesting that functional protective immune responses can in fact be primed.[44] It has been shown that patients who spontaneously clear acute HCV infections have an active endogenous T-cell response to HCV, whereas those who progress to chronicity have not.[6,7] This, obviously antiviral, T-cell response has been shown to be directed towards the NS3 protein.[6,7] In addition, NS3-specific T-cell responses are rarely found in patients with chronic HCV infections.[45–47]. Key factors, in basing a therapeutic HCV vaccine on the NS3 protein, are that NS3 is a large protein and encoded by a region within the HCV genome with a low genetic heterogeneity. This increases the chance that a vaccine-primed response will recognize the virus present in the host. Thus, if one were to combine existing antiviral therapies with NS3/4A-based therapeutic vaccination, the combined antiviral and immune activating effects may help to push the virus–host balance in favor of the host, resulting in a higher frequency of patients with a sustained virological response to therapy.

Materials and methods

Mice

Inbred BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were obtained from commercial vendors (Charles River, Uppsala, Sweden). The ethical committee for animal research at Karolinska Institutet had approved all animal experiments.

Synthetic peptides

20-mer peptides, corresponding to the complete NS3/4A sequence used as DNA immunogen, were synthesized by automated peptide synthesis as described previously.[48] Additional 9-mer peptides used in the fine mapping of the CTL epitope in H-2^b mice were produced by similar procedures.

Recombinant NS3 ATPase/helicase domain protein

The recombinant NS3 (rNS3) protein was kindly provided by Darrell L Peterson (Department of Biochemistry, Commonwealth University, VA). The production of recombinant NS3 protein (not including NS4A) in *Escherichia coli* has been described in detail previously.[49] Prior to use, the rNS3 protein was dialyzed overnight against PBS and sterile filtered.

HCV NS3 and NS3/NS4A DNA plasmids and in vitro mutagenesis

A full-length NS3 and NS3/NS4A gene fragment was amplified from a patient infected with HCV genotype 1b as previously described.[15] The NS3 and NS3/4A genes were inserted into the eukaryotic expression vector pVAX1 (Invitrogen, San Diego, CA, USA). For amplification of NS3, the forward primer 5′-GTG GAA TTC **ATG** GCG CCT ATC ACG GCC TAT-3′ and the reverse primer 5′-CCA CGC GGC CGC GAC GAC **CTA** CAG-3′ were used to introduce *Eco*RI and *Not*I restriction sites (underlined). The engineered translation initiation and stop codon is shown in bold. For amplification of NS3/NS4A, the forward primer 5′-GTG GAA TTC **ATG** GCG CCT ATC ACG GCC TAT-3′ and the reverse primer 5′-CCC TCT AGA **TCA** GCA CTC TTC CAT TTC ATC-3′

were used to introduce *Eco*RI and *Xba*I restriction sites (underlined). All expression constructs were sequenced to ensure correct sequence and reading frame. The size of the constructs was analyzed by PCR and restriction enzyme cleavage.

A mutant NS3/4A (mNS3/4A) gene where the amino terminal serine residue was mutated to a proline was produced by site-directed *in vitro* mutagenesis (Quik-Change, Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA, USA). Forward (5′-CTG GAG GTC GTC ACG CCT ACC TGG GTG CTC GTT-3′) and reverse primer (5′-AAC GAG CAC CCA GGT AGG CGT GAC GAC CTC CAG-3′) containing the mutation were used to introduce the mutation in the plasmid and generated the mNS3/4A-pVAX1 vector. The mutant construct was sequenced to control the desired mutation sequence and to ensure correct reading frame. The plasmid was also tested by an *in vitro* transcription and translation assay to ensure that the gene was intact and could be translated.

Plasmid DNA was grown and purified from *E. coli* through culturing colonies from LA/Kana plate in Luria–Bertani (LB) media containing 50 μg kanamycin/ml as previously described.[15,25] The purified plasmid DNA was dissolved in sterile phosphate-buffered saline (PBS) to a concentration of 1 mg/ml.

Semliki forest virus (SFV) vectors and transfection experiments

Baby Hamster kidney (BHK)-21 cells were maintained in complete BHK medium supplemented with 5% FCS, 10% tryptose phosphate broth, 2 mM glutamine, 20 mM Hepes and antibiotics (streptomycin 10 μg/ml and penicillin 100 IU/ml).

The sequence encoding NS3, NS3/4A and mNS3/4A was isolated by PCR as SpeI-BStB1 fragments and inserted into the *Spe*1-*Bst*B1 site of pSFV10Enh containing a 34 aa long translational enhancer sequence of capsid followed by the FMDV 2a cleavage peptide.[50,51]

Packaging of recombinant RNA into rSFV particles was done using a two-helper RNA system.[50,51] In brief, BHK cells were cotransfected with recombinant RNA and two helper RNAs, one of which codes for the SFV capsid protein and the other for the envelope proteins. After 48 h incubation, medium containing recombinant virus stock was harvested and purified.[52] Indirect immunofluorescence of infected BHK cells was performed to determine the titre of the recombinant virus stocks.[50,51]

Analysis of expression of NS3 protein from rSFV particles

Metabolic labelling of rSFV-infected cells with [^{35}S]methionine has been previously described.[50,51] Briefly, BHK cells were infected with rSFV particles at an MOI of 5. After 15 h the growth medium was replaced with methionine-free MEM for 30 min prior to the addition of fresh medium containing 75 μCi/ml [^{35}S] methionine. After a 15 min labelling period, the cells were incubated further for various times in medium containing unlabelled methionine. Supernatants were collected and the cells lysed with Nonidet P-40 buffer containing 100 mM iodoacetamide.

Protein sample preparation and analysis

Cell lysates were analyzed by immunoprecipitation followed by SDS-PAGE, as described previously.[50,51] Cell lysates were immunoprecipitated with protein A sepharose and anti-NS3 monoclonal antibody (kindly provided by G Inschauspé, Lyon, France) O/N at 4°C. The washed pellets were resuspended in SDS sample buffer, and heated at 95°C for 5 min prior to SDS-PAGE analysis on 10% acrylamide reducing gel.

Immunofluroescence

Indirect immunofluorescence of rSFV-infected BHK cells was carried out to detect the expression of NS3 protein. BHK cells were infected with rSFV-NS3, NS3/4A or mNS3/4A at an MOI of 5. After 16, 18 or 24 h growth, the cells were fixed in methanol and the protein expression detected by incubation of the cells with anti-NS3 monoclonal ab (kindly provided by G Inschauspé, Lyons, France) and subsequently anti-mouse IgG FITC (Sigma).

In vitro translation assay

To ensure that the NS3 and NS3/4A genes were intact and could be translated, an *in vitro* transcription assay using the prokaryotic T7 coupled reticulocyte lysate system (TNT; Promega, Madison, WI, USA) was performed as previously described.[15,25]

Immunization protocols

Groups (5–20 mice/group) of female BALB/c (H-2^d) or C57BL/6 (H-2^b) mice, 4–8 weeks old, were immunized by needle injections of 100 μg of plasmid DNA encoding individual or multiple HCV proteins. Plasmid DNA in PBS was given i.m. in the *tibialis anterior* (TA) muscle.[53] Where indicated in the text, the mice were injected i.m. with 50 μl/TA of 0.01 mM cardiotoxin (Latoxan, Rosans, France) in 0.9% sterile saline NaCl, 5 days prior to DNA immunization. The mice were boosted at 4-week intervals.

For gene gun-based immunizations, plasmid DNA was linked to gold particles according to protocols supplied by the manufacturer (Bio-Rad Laboratories, Hercules, CA, USA). Prior to immunization, the injection area was shaved and the immunization was performed according to the manufacturer's protocol. Each injection dose contained 4 μg of plasmid DNA. The mice were boosted with the same dose at monthly intervals.

Peptide immunizations were performed using 100 μg peptide mixed with complete Freunds adjuvant (1:1), and injected subcutaneously (s.c.) in the base of the tail.

ELISA for detection of murine anti-HCV NS3 antibodies

Serum for antibody detection and isotyping was collected every second or fourth week after the first immunization by retro-orbital bleeding of isofluorane-anesthetized mice. The enzyme immunoassays were performed as previously described.[15,28]

Cell lines

The SP2/0-Ag14 myeloma cell line (H-2^d) was maintained in DMEM medium supplemented with 10% fetal calf serum (FCS; Sigma Chemicals, St Louis, MO, USA), 2 mM L-glutamin, 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin, 1 mM non-essential amino

acids, 50 μM β-mercaptoethanol and 1 mM sodium pyruvate (GIBCO-BRL, Gaithesburgh, MD, USA). SP2/0-Ag14 cells with stable expression of NS3/4A were generated by transfection of SP2/0 cells with the linearized NS3/4A-pcDNA3.1 plasmid using the Super-Fect (Qiagen GmbH, Hilden, FRG) transfection reagent. The transfection procedure was performed according to the manufacturer's protocol. Transfected cells were cloned by limiting dilution and selected by the addition of 800 μg geneticin (G418)/ml complete DMEM medium. Expression of NS3/4A was confirmed by reversed transcription PCR and by a capture EIA using a monoclonal antibody to NS3.[25]

RMA-S cells (a kind gift from Professor Klas Kärre) were maintained in RPMI1640 medium supplemented with 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were grown in a humidified 37°C, 5% CO_2 incubator.

Detection of CD4+ proliferative responses to NS3

The detection of proliferative responses to NS3 followed previously described protocols.[15,28] In brief, groups of mice were immunized with 100 μg NS3-pVAX1 or NS3/4A-pVAX1 in TA muscles. Splenocytes were harvested 13 days later, single-cell suspensions were prepared and the cells were incubated with serial dilutions of rNS3. The cells were incubated with or without rNS3 for 4 days, and for the last 24 h ^{3}H-labelled thymidine (TdR) was added. A liquid scintillator was used to measure the uptake of radioactive thymidine.

In vivo challenge with the NS3/4A-expressing SP2/0 myeloma

In vivo challenge of immunized mice with the NS3/4A-expressing SP2/0 myeloma was performed according to the method described by Encke *et al*.[10] In brief, groups of BALB/c mice were immunized with different immunogens at weeks 0, 4, 8, 12 and 16 as described. In all, 2 weeks after the last immunization, 1 or 2×10^6 NS3/4A-expressing SP2/0 cells were injected s.c in the right flank. The kinetics of the tumor growth was determined by measuring the tumor size through the skin at days 6 to 14. The mean tumor sizes were calculated and the AUC values from the groups were compared using ANOVA. At day 14, all mice were killed, the tumors were removed, paraffin embedded, and sectioned. Tumor sections of 4 μm thickness were mounted on slides and stained with hematoxylin–eosin dye according to standard procedures. The amount of T-cell infiltration in the tumor was determined by staining with the anti-CD3 antibody (Dako, Denmark). A pathologist who was blinded as to which group the section belonged, analyzed the histological appearance of the tumors.

Immunohistochemistry

Tumor tissue was placed in formalin and embedded in paraffin; 4 μm sections were prepared. Paraffin-embedded sections were pretreated with an avidin–biotin blocking kit (Vector, Vector Laboratories, Burlingame, CA, USA) and then immunostained with an anti-CD3 antibody. For detection, biotinylated immunoglobulins, followed by avidin–biotin peroxidase (Vector) were used. Microwave pretreatment was also used for CD3 immunostaining.

Antibodies and MHC:Ig fusion protein

All monoclonal antibodies and MHC:Ig fusion proteins[32] were purchased from BDB Pharmingen (San Diego, CA, USA); anti-CD16/CD32 (Fc-block™, clone 2.4G2), FITC-conjugated anti-CD8 (clone 53-6.7), FITC-conjugated anti-H-2K^b (clone AF6-88.5), FITC-conjugated anti-H-2D^b (clone KH95), recombinant soluble dimeric mouse H-2D^b:Ig, PE-conjugated Rat-α Mouse IgG1 (clone X56), FITC-conjugated anti-BrdU (clone B44), PE-conjugated anti-CD69 (clone H1.2F3), Cy-Chrome™ conjugated anti-CD45R/B220 (clone RA3-682).

Peptide stabilization assay

To identify NS3/4A peptides able to bind to MHC class I molecules, we tested overlapping 20-mer peptides (in total, 69 different peptides with 10 aa overlap) for binding to MHC class I using the RMA-S stabilization assay described previously.[33,34] Briefly, 1×10^6 RMA-s cells were incubated in RPMI1640 medium supplemented with 10% FCS, 2 mM L-glutamine and 10 mM HEPES, for 16–20 h with 0.3 mM of individual 20-mer peptides at room temperature (~21°C). Cells were then washed and stained for 30 min on ice with an optimal concentration (1μg/10^6) of FITC-conjugated anti-H-2K^b or anti-H-2D^b antibodies. Cells were resuspended in PBS/1% FCS (FACS buffer) containing 0.5 μg/ml of propidium iodine (PI; Sigma). The H-2K^b and H-2D^b expression on live cells (PI negative) were analyzed by FACS. In order to determine the optimal aa sequence of one selected H-2D^b binding 20-mer peptide, nine aa long peptides with an eight aa overlap were synthesized and evaluated for H-2D^b binding. Varying peptide concentrations (0.01–100 μM) were used and peptide-loaded RMA-S cells were chased at 37°C for 45 min prior to staining with anti-H-2D^b antibodies in order to reduce nonspecific background (data not shown).

Detection of NS3/4A-specific CTL

Spleen cells from DNA-immunized BALB/c mice were resuspended in complete DMEM medium. *In vitro* stimulation was carried out for 5 days in 25-ml flasks at a final volume of 12 ml, containing 5 U/ml recombinant murine IL-2 (mIL-2; R&D Systems, Minneapolis, MN, USA). The restimulation culture contained a total of 40×10^6 immune spleen cells and 2×10^6 irradiated (10 000 rad) syngenic SP2/0 cells expressing the NS3/4A protein. After 5 days *in vitro* stimulation, a standard ^{51}Cr-release assay was performed. SP2/0 cells and SP2/0 cells expressing the NS3/4A protein served as targets, and were labelled for 1 h with 20 μl of ^{51}Cr (5 mCi/ml) and then washed three times in PBS. Serial dilutions of effector cells were incubated with 5×10^3 ^{51}Cr-labelled target cells/well. After a 4 h incubation at 5% CO_2, 37°C, 100 μl of supernatant was collected and the radioactivity was determined by a γ-counter.

Spleen cells from peptide-immunized mice (12 days post-immunization) or naïve mice were resuspended in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin, 1 mM nonessential aa, 50 μM β-mercaptoethanol and 1 mM sodium pyruvate. *In vitro* stimulation was carried out for 5 days in 25-ml flasks in a total volume of 12 ml, containing 25×10^6 spleen cells and 25×10^6 irradiated (2000 rad) syngeneic splenocytes.

The restimulation was performed in the presence of 0.05 μM NS3 H-2D^b binding peptide (sequence GAVQNEVTL) or irrelevant H-2D^b peptide (sequence KAVYNFATM). After 5 days *in vitro* culture, a ^{51}Cr-release assay was performed as described above. RMA-S cells and RMA-S cells pulsed with 50 μM peptide for 1.5 h at +37°C prior to ^{51}Cr labelling served as targets.

Quantitation of NS3-specific CTLs by flow cytometry

The frequency of NS3/4A-peptide specific CD8+ T cells was analyzed by *ex vivo* staining of spleen cells from NS3/4A DNA-immunized mice with recombinant soluble dimeric mouse H-2D^b:Ig fusion protein. Spleen cells (10^6) resuspended in 100 μl PBS/1% FCS (FACS buffer) were preincubated with 1 μg/10^6 cells of Fc-blocking antibodies on ice for 15 min. The cells were then incubated on ice for 1.5 h with either 2 μg/10^6 cells of H-2D^b:Ig preloaded for 48 h at +4°C with 160 nM excess of NS3-derived peptide (sequence GAVQNEVTL) or 2 μg/10^6 cells of unloaded H-2D^b:Ig fusion protein. The cells were then washed twice in FACS buffer and resuspended in 100 μl FACS buffer containing 10 μl/100 μl PE-conjugated rat-α mouse IgG1 secondary antibody, and incubated on ice for 30 min. The cells were then washed twice in FACS buffer and incubated with 1 μg/10^6 cells of FITC-conjugated α-mouse CD8 antibody for 30 min. The cells were then washed twice in FACS buffer and resuspended in 0.5 ml FACS buffer containing 0.5 μg/ml of PI. Approximately 200 000 events from each sample were acquired on a FACS Calibur (BDB) and dead cells (PI positive cells) were exluded in the analysis.

In vitro B cell activation and proliferation assay

BALB/c splenocytes (2×10^6/ml) in RPMI 1640 medium, 10% FCS were stimulated for 24 or 48 h with 5 μg/ml pVAX1 vector or 5 μg/ml NS3-pVAX1 DNA or 5 μg/ml NS3/4A-pVAX1 DNA. Cells grown in medium only served as a negative control, and 1 μg/ml LPS (Sigma Chemicals, St. Louis, MO, USA) and 1.3 μg/ml of a phosphorothioate-modified oligodeoxynucleotide (ODN; Cybergene AB, Sweden) termed CpG-1826[38] served as positive controls. During the last 4 h of culture, bromodeoxyuridine (BrdU; Sigma Chemicals) was added at a final concentration of 10 μM. At the end of the culture, cells were centrifuged and washed two times in PBS/1% FCS. After the final wash, cells were preincubated with 2.4G2 mAb (1 μg/10^6 cells in PBS/1% FCS) for 20 min at +4°C. Cells were then washed as described above. Thereafter, cells were stained with PE-conjugated anti-CD69 antibody and Cy-Chrome™-conjugated anti-CD45R/B220 antibody for 30 min at +4°C. Cells were then washed as described above. Thereafter, cells were fixed and permeabilized by adding 100μl Cytofix/Cytoperm™ solution (included in Cytofix/Cytoperm Plus kit; BDB Pharmingen) per well and incubated for 20 min at +4°C. Cells were thereafter washed in Perm/Wash™ solution (included in Cytofix/Cytoperm Plus kit). Cells were stained with 1:10 of FITC-conjugated anti-BrdU antibody diluted in Perm/Wash™ solution supplemented with 2.5 μl/ml of a 2000 U/ml (50 mg/ml PBS) DNase I purchased from Boehringer Mannheim (Mannheim, Germany). Cells were incubated for 1 h in the dark at room temperature and then washed twice in Perm/Wash™ solution and resuspended in PBS/1% FCS. Samples were analyzed on a FACS Calibur™ (BDB), and the percentage of B cells (CD45R/B220 gate) positive for CD69 and BrdU were calculated using the CellQuest™ (BDB) program.

Statistical analysis

Fisher's exact test was used for frequency analysis and Mann–Whitney *U*-test was used for comparing values from two groups. Kinetic tumor development in two groups of mice were compared using the area under the curve (AUC). AUC values were compared using analysis of variance (ANOVA). The calculations were performed using the Macintosh version of the StatView software (version 5.0).

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